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Quantification of *Frankia* Strains and Other Root-Associated Bacteria in Pure Cultures and in the Rhizosphere of Axenic Seedlings by High-Performance Liquid Chromatography-Based Muramic Acid Assay

R. RÖNKKÖ,[1]* T. PENNANEN,[1] A. SMOLANDER,[2] V. KITUNEN,[2] H. KORTEMAA,[3] AND K. HAAHTELA[1]

Department of General Microbiology[1] and Department of Plant Biology,[3] FIN-00014 University of Helsinki, and Department of Forest Ecology, Finnish Forest Research Institute, FIN-01301 Vantaa,[2] Finland

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Application of a high-performance liquid chromatography-based muramic acid assay with precolumn fluorescence derivatization to quantification of root-associated bacteria was studied both in pure cultures and in the rhizosphere of axenic *Festuca rubra* seedlings. Quantities of muramic acid from acid-hydrolyzed cells of *Frankia* strains, *Streptomyces griseoviridis*, *Enterobacter agglomerans*, *Klebsiella pneumoniae*, *Pseudomonas* sp., and *Bacillus polymyxa* were mostly proportional to the respective cell protein and carbon quantities, but in some strains, culture age and particularly sporulation affected these ratios considerably. The muramic acid/cell protein ratio was generally 2 to 4 times higher in strains of the two actinomycete genera, *Frankia* and *Streptomyces*, than in the rest of the strains. Quantification of *Frankia* strains, *S. griseoviridis*, *E. agglomerans*, and *Pseudomonas* sp. was also attempted from the rhizosphere of *F. rubra* seedlings which had been inoculated with pure cultured bacteria and incubated briefly. It was possible to quantify *Frankia* cells by use of the muramic acid assay from both the root and the growth medium, whereas cells of the rest of the bacterial genera could only be detected in the medium. The detection limit for muramic acid was about 10 ng/ml hydrolysis volume, and from the *Festuca* rhizosphere, 28 to 63% of the muramic acid in the *Frankia* inoculum was recovered.

Quantification of hyphal microbes, e.g., actinomycetes and fungi, is more complicated than that of unicellular ones because separation and counting of individual cells from the hyphae is usually impossible. Use of conventional viable counting methods, such as dilution plating or most probable number in liquid culture, require that the hyphae are first homogenized and then counted as viable units, which may vary in size depending on the homogenization process and the organism itself. Another problem is the slow growth rate of members of some microbial groups, which makes the viable count methods far from immediate and makes them susceptible to contaminants. These problems have been partially overcome by quantification methods which measure the amount or activity of some cellular component. For example, cell protein and carbon have been used to quantify microbes in pure cultures (1, 15) and in numerous chemical and biochemical assays to estimate the total microbial biomass in soil (22, 23).

Both fungi and bacteria contain unique cellular components which are not encountered in other organisms and are thus suitable for specific quantification. Muramic acid (MA), which is encountered in the cell walls of almost all bacteria, has been used for quantification of bacteria in ecological studies (2, 9, 11–14, 26). These studies have concentrated on the sediment, water, and soil bacterial biomass as a whole. The early MA assays used colorimetric (5, 11), enzymatic (12, 13), and gas-liquid chromatography (2) methods. Later, high-performance liquid chromatography (HPLC) with precolumn fluorescence derivatization was adopted (9, 14, 26), with greatly increased sensitivity and speed. In this method, acid hydrolysis is used to release, from the cell wall, the MA, which is then derivatized with *o*-phthaldialdehyde to a fluorescent compound and quantified by HPLC. Even though this method has been technically well described, the respective biological knowledge still remains relatively poor and scattered. However, thorough studies with different bacterial genera and strains concerning the quantitative relations between MA and the other cell components, and possible alterations in these relations, are the prerequisite for specific applications of this method.

Organisms of the actinomycete genus *Frankia*, best known as the N_2-fixing root nodule symbiont of actinorhizal plants, have slow growth and exhibit a hyphal morphology. For pure cultures of *Frankia* cells, a destructive total protein assay has been found to be the most accurate quantification method but packed cell volume and turbidity measurements have also been traditionally used (15). The nodule induction capability of *Frankia* strains has also been utilized in quantification, especially for *Frankia* organisms in soil (19, 24). In this bioassay, a homogenized *Frankia* sample is inoculated into the rhizosphere of a compatible host plant, and the number of evolving nodules gives an estimate of *Frankia* abundance (nodule-forming units). Despite the advantages of this method, such as its specificity for viable *Frankia* hyphae, it suffers from the same problems as the other viable counting methods and does not necessarily measure vegetative growth accurately.

In our previous studies with *Frankia* strains and axenic seedlings, the abundance of *Frankia* hyphae in the rhizosphere was estimated with light and electron microscopy (16, 17). To supplement the microscopical methods, a specific and more accurate chemical quantification method for bacteria was sought. In this paper, we describe the application of HPLC-based MA assay with precolumn fluorescence derivatization to

* Corresponding author. Mailing address: Department of General Microbiology, P.O. Box 41 (Mannerheimintie 172), FIN-00014 University of Helsinki, Finland.

the quantification of root bacteria, with special emphasis on *Frankia* strains, in pure cultures and in the rhizosphere of axenic seedlings.

MATERIALS AND METHODS

Bacteria. *Frankia* strains Ai17 (18) and Ai1a UHF 01111 (7, 25) were cultured in 150-ml aliquots of propionate-Casamino Acids mineral medium, and the bacteria were collected, washed, and homogenized as described earlier (19). *Streptomyces griseoviridis* (20, 21) was grown in 100-ml aliquots of GYM medium (4 g of glucose, 4 g of yeast extract, and 10 g of malt extract per liter, pH 7.3) and treated similarly to *Frankia* strains. The associative N_2-fixers, *Enterobacter agglomerans* Am, *Klebsiella pneumoniae* As, and *Pseudomonas* sp. strain Dc (3, 4), were cultured in 200-ml aliquots of malate liquid medium (3), collected by centrifugation (8,000 × *g*), and washed in sterile water. *Bacillus polymyxa* DSM 36 was cultured in 150- to 300-ml aliquots of modified malate medium (100 mg of yeast extract, 5 g of glucose, 2 g of sucrose, 5 μg of biotin, and 10 μg of *p*-aminobenzoic acid were supplemented per liter) and collected as described above. All the bacteria were incubated at 28°C in static cultures, except *S. griseoviridis* and *B. polymyxa*, which were grown with shaking (120 rpm). After being washed, the bacteria were resuspended in a 10-ml volume, from which samples were taken for quantification and inoculation of *Festuca rubra* seedlings. Samples were stored at −20°C if not assayed immediately. All the bacterial suspensions were quantified by protein and MA assays (see below). The associative N_2 fixers and *B. polymyxa* were additionally quantified by viable count plating, and some *Frankia* cultures were quantified by organic carbon analysis (see below).

Growth curve of *Frankia* strain Ai1a. A set of infusion bottles, each containing 40 ml of propionate-Casamino Acids medium, were inoculated with a small volume of homogenized *Frankia* strain Ai1a suspension equivalent to 20 μg of cell protein. The cultures were incubated at 28°C statically but shaken briefly once a week. Three replicate bottles were taken from the set after 1, 2, 4, 6, 9, and 13 weeks of incubation, and the bacteria were collected by filtration and assayed for cell protein and MA (see below). The pH of the culture medium was measured after removal of bacteria.

Plant germination, inoculation, and recovery of bacteria. Red fescue (*F. rubra*) seedlings were germinated from surface-sterilized seeds and grown axenically in glass tubes with 10 ml of mineral medium and glass beads as support material as described earlier (16). Control tubes without plants were prepared and treated in a similar way to the plant tubes. Four-week-old *Festuca* seedlings were inoculated with *Frankia* strain Ai17 and grown for a further 2 weeks. In another experiment, 12-week-old *Festuca* seedlings were inoculated with *Frankia* strain Ai17, *S. griseoviridis*, *E. agglomerans*, or *Pseudomonas sp*, and incubated further briefly (16 to 20 h) to allow bacterial attachment. The seedlings were then carefully removed from the tubes, and the roots were dissected into tared 4-ml screw-capped ampules and weighed (root fresh weight). For the MA assay, 2 ml of 6 M HCl was added to each ampule. The culture medium was collected separately from the tubes, which were further rinsed three times with sterile water, together with the glass beads, to remove all bacteria. The pool of medium and rinsing solutions was filtered through reusable filter cartridges (Sartorius; filter diameter, 25 mm and pore size, 0.45 μm), and the bacteria were washed from the filter into a small volume (0.6 ml) of 0.1 M HCl. An equal volume of 12 M HCl was added to the solution in order to raise the HCl concentration to 6 M for the MA assay.

Protein assay. Samples from the bacterial suspensions were centrifuged, and the pellets were resuspended in 1.5 to 2 ml of 0.1 M HCl. The samples were hydrolyzed by boiling and sonicated, and the cell protein was quantified with the Bio-Rad assay as described earlier (19). Those *Frankia* samples from which organic carbon was additionally assayed (see below) were also passed through 0.2-μm pore size membrane filters (Minisart NML; Sartorius) before analysis to remove all particulate matter.

Organic carbon analysis. Samples from *Frankia* suspensions were hydrolyzed, sonicated, and filtered similarly to the protein assay samples (see above), and the dissolved organic carbon was measured with a total organic carbon analyzer (TOC-5000; Shimadzu) with potassium hydrogen phthalate as standard.

MA assay. The MA assay is based on the method used by Lindroth and Mopper (8) for amino compounds and on the further application of this method for MA and bacterial quantification (14).

(i) HPLC. The HPLC apparatus consisted of a Merck-Hitachi AS-4000 automated injector, an L-6200 gradient pump and D-2500 integrator, and a Shimadzu RF-S30 fluorescence detector (excitation wavelength, 310 nm; emission, 410 nm). An ODS-Hypersil column (C18; 5 μm, 250 by 4 mm) and guard column (C_{18}; 5 μm, 20 by 4 mm) were used.

(ii) Eluents, derivatization reagent, and MA standard. Eluent A was 0.05 M Na acetate (pH 7.5) with 2% (vol/vol) tetrahydrofuran, and eluent B was 80% (vol/vol) methanol and 20% (vol/vol) 0.05 M Na acetate (pH 7.5). The OPA reagent (*o*-phthaldialdehyde and mercaptoethanol in 0.4 M borate buffer, pH 9.5) was prepared as described by Lindroth and Mopper (8). A stock solution containing 1 mg of MA per 1 ml of 0.1 M HCl, from which standards for the HPLC analysis were diluted, was prepared (final concentrations, 10 to 2,000 ng/ml). *o*-Phthaldialdehyde and 2-mercaptoethanol were from Merck, and MA was from Sigma.

(iii) Sample preparation. Bacterial pure culture samples were first lyophilized (Christ L-1 lyophilizer; B-Braun) and resuspended in 2 ml of 6 M HCl. All samples (pure cultures, *Festuca* roots, and growth medium filtrates) were hydrolyzed in 6 M HCl at 105°C for 3 h. The root samples were centrifuged after hydrolysis to remove the plant debris. Prior to hydrolysis, MA (0.1 μg/ml hydrolysis volume) was added into a few replicates as an internal standard. Hydrolysates were stored at −20°C until HPLC analysis.

(iv) HPLC procedure. A 100-μl sample of a hydrolysate or MA standard solution was pipetted into an HPLC vial. The autoinjector was programmed to add 96 μl 6 M NaOH and 490 μl of OPA reagent into the vial (final pH, 8.0 to 8.5), mix the sample briefly, and incubate it statically for 2 min. A 100-μl sample of the derivatized mixture was then injected into the column, in which the flow rate was 1.2 ml/min. A linear elution gradient from 100% eluent A at 0 min to 75% eluent A at 16 min and further to 100% eluent B at 20 min was used. Finally, the column was washed for 5 min with eluent B.

Statistics. The data were treated with one-way analysis of variance and Tukey's test ($P < 0.05$). Logarithmic transformation ($\log_{10}$) was applied for most of the data in Tables 1 and 2.

RESULTS

General evaluation of MA assay. HPLC runs with MA standards showed a very good linearity over a broad concentration range (e.g., a standard curve including concentrations of 10, 30, 50, 80, 100, 200, 600, and 1,000 ng/ml gave a linear correlation of 0.999, $P < 0.1\%$). The retention time of MA in our system was 15 ± 1 min (Fig. 1). The background from

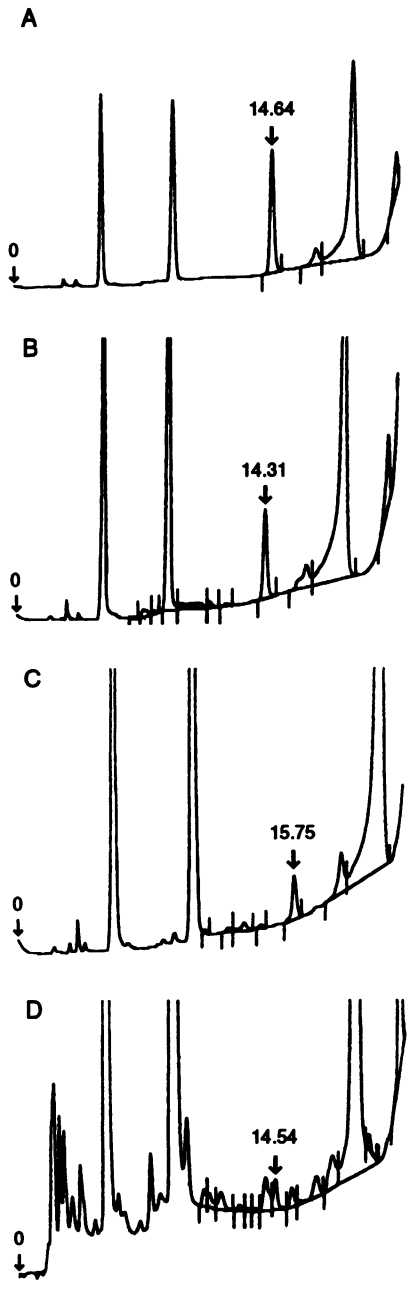

FIG. 1. HPLC runs of an MA standard (200 ng/ml) (A), *Frankia* strain Ai1a pure culture (B), filtrate from *Festuca* growth medium inoculated with *Frankia* strain Ai17 (C), and *Festuca* root inoculated with *Frankia* strain Ai17 (D). The injection points (0) and MA peaks (retention time shown in minutes) are indicated with arrows.

other bacterial compounds was minimal in all pure culture samples (Fig. 1B), and thus an internal standard was not necessary. This was also true for samples containing filtered *Festuca* growth medium (Fig. 1C) from inoculation experiments. In the root samples, which originally contained solid plant material, a considerably higher background was observed (Fig. 1D), and because of an unidentified compound eluting at only a slightly shorter retention time, replicates spiked with MA were used to ensure reliable identification. The detection limit for MA was found to be about 5 ng per ml hydrolysis volume for MA standards and 10 ng/ml for pure culture and root samples. Even though smaller amounts were still detectable, the quantification was not reliable.

The MA concentration was found to decrease in hydrolyzed samples during prolonged storage even at −20°C. Therefore, the samples were always analyzed within a few days after hydrolysis. Repeated runs with the same sample usually showed only nominal variation in quantities, and thus the quantitative differences observed between samples were mainly of biological origin. The correct pH (8.0 to 8.5) during the derivatization was crucial for the assay, and occasional malfunctions of the autosampler (no NaOH added) caused failures in MA detection. These failures could, however, be easily observed and corrected with repeated runs.

Quantification of bacteria in pure cultures. Bacterial pure cultures were quantified with both MA and protein assays, and viable count was also done for the unicellular strains. The quantification results are presented in Table 1, as well as the MA/protein and MA/CFU count ratios. With the 2-ml hydrolysis volume used, reliable quantification of MA was reached with samples containing at least 20 ng of MA, equalling about 2.5 μg of *Frankia* strain Ai17 cell protein. Overall, with sample sizes from 5 to 170 μg of cell protein (Table 1), the MA assay gave a relatively constant MA measurement for each strain, as indicated by the MA/protein and MA/CFU count ratios.

The MA contents varied between different strains (Table 1). The MA/protein ratio was considerably higher in the actinomycete strains, genera *Frankia* and *Streptomyces*, than in the unicellular bacteria. In the strongly sporulating *Streptomyces* culture, the ratio was about 30 times higher than in the respective nonsporulating culture. In the unicellular bacteria grown for 24 h, the MA/cell protein ratios were almost equal in *Bacillus*, *Enterobacter*, and *Klebsiella* cultures but somewhat lower in *Pseudomonas* cultures. The MA/CFU count ratios varied considerably between all of these strains. An older *Bacillus* culture (7 days) was also analyzed, and the MA/protein ratio was significantly lower and the MA/CFU count ratio was higher than in the respective 24-h culture.

In order to monitor the MA content in the early growth phases of *Frankia* cultures, a growth curve of strain Ai1a in PC medium was prepared, extending from inoculation to 13 weeks. The cell protein and MA quantities of Ai1a are plotted versus incubation time in Fig. 2. Both the MA and protein assays gave similar growth curves, and the MA/protein ratio was observed to be relatively constant during the 13-week period (Fig. 2). A gradual pH increase was measured in the cultures, from 6.7 in the original medium to 8.0 in the 13-week-old cultures.

The MA content and, for comparison, cell protein and organic carbon were also determined in older *Frankia* Ai1a and Ai17 cultures (3, 34, 43, and 106 weeks; the cultures were started neither simultaneously nor from the same inoculum batch). Cell protein was assayed both with and without filtration after hydrolysis, the former treatment being comparable with the organic carbon assay, in which prefiltering was necessary, and the latter with the MA assay. Table 2 shows the culture densities determined by the different methods and the ratios between them. The MA/protein and MA/organic carbon ratios were relatively similar in Ai1a cultures of different ages, except for the somewhat lower value in the 43-week-old culture. More variation was seen in Ai17 cultures, for which the highest ratio was encountered in the youngest culture and the lowest was encountered in the 43-week-old Ai17 culture, which exhibited unusually poor growth, as indicated by the low culture density (Table 2). Whether this was

TABLE 1. Quantification of pure cultured bacteria by cell protein, viable count, and MA assays

Bacterial type and strain (age of culture)	Suspension vol (μl)	Amt of cell protein (μg)	VC[a] (10^6 CFU)	MA (ng)[b]		
				Total	Per μg of cell protein	Per 10^6 CFU
Gram positive						
Frankia strain Ai17 (132 days)	8	5.0	—[c]	100	20 (24 ± 3 a)	—
	30	19	—	480	26	—
	100	63	—	1,600	26	—
S. griseoviridis (25 days, nonsporulating)	0.5	7.8	—	110	14	—
S. griseoviridis (42 days, sporulating)	0.5	0.65	—	290	450	—
B. polymyxa (1 day)	10	15	5.0	93	6.1 (5.9 ± 0.2 b)	19 (18 ± 1 a)
	40	61	20	360	6.0	18
	100	150	50	840	5.6	17
B. polymyxa (7 days)	10	17	0.17	29	1.7 (1.4 ± 0.2 c)	180 (150 ± 20 b)
	40	70	0.66	84	1.2	130
	100	170	1.7	230	1.3	140
Gram negative						
E. agglomerans Am (1 day)	30	37	440	230	6.3 (6.2 ± 0.2 b)	0.55(0.54 ± 0.02 c)
	60	74	880	480	6.5	0.55
	100	120	1,500	740	6.0	0.51
K. pneumoniae As (1 day)	15	34	190	200	6.0 (5.9 ± 0.2 b)	1.1 (1.1 ± 0.0 d)
	30	67	370	400	5.9	1.1
	60	130	740	760	5.6	1.0
Pseudomonas sp. strain Dc (1 day)	8	17	16	770	4.4 (4.2 ± 0.2 d)	4.8 (4.6 ± 0.2 e)
	30	65	60	270	4.2	4.5
	60	130	120	530	4.1	4.4

[a] Viable count.
[b] Each value represents the total sample biomass added to the hydrolysis mixture in the MA assay. Values in parentheses are means of the three sample sizes (each set of three rows) ± standard deviations. Means within a column followed by the same letter do not differ significantly ($P < 0.05$).
[c] —, not measurable.

due to the inoculum or to the medium batch is not known. The MA/protein and MA/organic carbon ratios of Ai1a cultures were always at least twice those of Ai17 cultures of equivalent age.

Quantification of bacteria in the rhizosphere of *Festuca* seedlings. In situ quantification of *Frankia* strain Ai17, *S. griseoviridis*, *E. agglomerans*, and *Pseudomonas* sp. in the rhizosphere of axenic 12-week-old *F. rubra* seedlings was attempted with the MA assay. After a short incubation (1 day), the bacteria attached to the root and in the growth medium were assayed separately and, for comparison, viable counts of *Enterobacter* and *Pseudomonas* organisms were also made. Detectable amounts of MA from both the root and growth medium samples inoculated with 5 and 25 μg (cell protein) of the *Frankia* strain were found. The results were somewhat less reliable with the smaller inoculum size, as indicated by the lower recovery and greater variation in the distribution of MA between the root and medium (Table 3). The majority of the *Frankia* MA recovered (63% with the 5-μg and 71% with the 25-μg inoculum) was found to be root attached. Calculated on a root fresh weight basis, the root-attached MA amount was over 10 times higher with the larger inoculum than with the smaller one. With the other three bacteria, MA was detected in the growth medium but not in the root samples, and the recoveries were rather low (Table 3). Viable counts of *Enterobacter* and *Pseudomonas* strains were obtained from both the roots and the growth medium, but the root-attached population was only a small fraction of the bacteria counted in the medium. The total recovered viable count of the *Enterobacter* strain was similar to the original inoculum size, whereas the *Pseudomonas* culture, which was inoculated at 1/10 the cell density, showed a net increase in viable cell numbers in the rhizosphere.

Quantification by MA assay was also tested with 4-week-old *Festuca* seedlings inoculated with *Frankia* strain Ai17 and incubated for a further 2 weeks before recovery. Consistent quantification was achieved with both inoculum sizes (5 and 25 μg of cell protein), but the amount recovered was less than 50% of the inoculum size (Table 3). In contrast to the experiment with 12-week-old seedlings, only a minority of the recovered MA was found to be root attached (11% with the smaller and 33% with the larger inoculum). However, calculated on a root fresh weight basis, the root-attached MA quantities were of the same magnitude as with the similarly treated older *Festuca* plants and the larger inoculum again caused markedly more colonization. From the plantless control tubes with *Frankia* strain Ai17 inocula (5 and 25 μg of cell protein), 15 and 115 ng of MA were recovered, corresponding to 27 and 54%, respectively, of the MA in the original inoculum.

DISCUSSION

The HPLC-based MA assay was found to be a reliable and relatively sensitive method for bacterial quantification. For bacterial pure cultures, the sensitivity was more than adequate and could be adjusted by variation of the hydrolysis volume with respect to the sample biomass. In bacterial samples also containing plant material, the volume could not be adjusted as freely because of the physical size of roots and

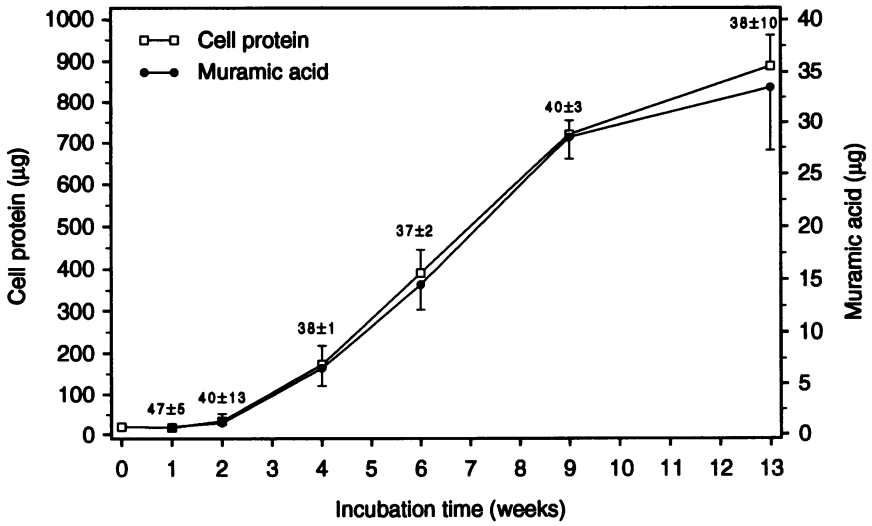

FIG. 2. Growth curve of *Frankia* strain Ail a assayed as total cell protein and MA per culture. The inoculum size was 20 μg of cell protein, and the culture volume was 40 ml of propionate-Casamino Acids medium. Error bars indicate the standard deviations ($n = 3$), and the numbers above the datum points indicate the MA/cell protein ratio (nanograms per microgram). No significant differences ($P < 0.05$) between the ratios were observed in analysis of variance.

additional acid consumption. This may be a problem in rhizosphere studies if the root-attached bacterial population is not very abundant, as was seen in the experiments with strains of *Enterobacter*, *Klebsiella*, and *Streptomyces* in which the levels of root-associated MA were below the detection limit. If the rhizosphere population is examined as a whole, without separating the root-attached and free bacteria in the growth medium, additional sensitivity may be achieved by hydrolyzing and analyzing the two pools together. Alternatively, the bacteria may be detached from the roots by mechanical or chemical means and analyzed with minimal background from plant material.

The reliability and variability of the MA assay was evaluated by comparison of the results with the values obtained by other quantification methods. Cell protein and organic carbon assays usually gave comparable estimates of bacterial abundance, even though the MA/cell protein and MA/organic carbon ratios varied in some cases with culture age and because of sporulation. Somewhat more variation was observed between the MA quantities and viable counts (unicellular strains). Even though these bacterial cultures were grown for the same time, they were probably in slightly different growth phases because of their individual growth rates and thus had different amounts of nongrowing cells and dead cell material.

The MA/organic carbon ratios, measured from *Frankia* Ail a and Ai17 strains of various ages, were of the same magnitude as the values presented in an early study of Moriarty (11). No *Frankia* strains were included in that study but, for a *Streptomyces* strain, the ratio was 52 (micrograms per milligram) and for two unicellular gram-positive strains it varied from 104 to 123. However, the MA was quantified by use of a colorimetric assay and the organic carbon was quantified by a dry combus-

TABLE 2. Culture densities of *Frankia* strains Ail a and Ai17 determined by cell protein, organic carbon, and MA assays, and ratios between the quantities obtained by these methods[a]

Strain and culture age (wk)	Culture density				Ratio		
	Cell protein (μg/ml)	Cell protein (filtered)[b] (μg/ml)	OC[c] (μg/ml)	MA (ng/ml)	MA/cell protein (ng/μg)[d]	MA/OC (ng/μg)[d]	Cell protein (filtered)/OC (μg/μg)
Ail a							
3	1.0 ± 0.1 a	0.18 ± 0.02 a	0.43 ± 0.14 a	44 ± 3 a	44 ± 3 a	104 ± 7 ab	0.48 ± 0.19 a
34	1.5 ± 0.1 b	0.30 ± 0.03 b	0.53 ± 0.02 a	65 ± 5 b	42 ± 4 a	123 ± 10 a	0.56 ± 0.04 a
43	3.7 ± 0.2 c	0.84 ± 0.08 c	1.4 ± 0.1 b	110 ± 20 c	30 ± 5 b	83 ± 13 b	0.62 ± 0.07 a
106	22 ± 1 d	7.9 ± 0.7 d	7.7 ± 0.5 c	830 ± 70 d	37 ± 3 ab	107 ± 9 ab	1.0 ± 0.2 b
Ai17							
3	2.0 ± 0.0 a	0.48 ± 0.01 a	0.73 ± 0.13 a	39 ± 1 a	20 ± 1 a	54 ± 2 a	0.67 ± 0.13 a
34	41 ± 1 b	18 ± 1 b	19 ± 0 b	560 ± 30 b	14 ± 1 b	29 ± 1 b	0.92 ± 0.03 a
43	3.3 ± 0.1 c	0.89 ± 0.07 c	1.2 ± 0.2 c	22 ± 1 c	6.6 ± 0.3 c	18 ± 1 c	0.74 ± 0.09 a
106	25 ± 1 d	9.0 ± 0.3 d	11 ± 0 d	320 ± 0 d	13 ± 0 b	29 ± 0 b	0.82 ± 0.04 a

[a] Means ± standard deviations ($n = 3$). Values for the same strain with a common letter do not differ significantly ($P < 0.05$). See also footnote *d*.
[b] Hydrolysates passed through 0.2-μm pore size membrane filters before assay.
[c] OC, organic carbon.
[d] For values in these columns only, there is a significant difference ($P < 0.01$) between the two strains.

TABLE 3. Inoculum sizes and recovery of bacteria from the rhizosphere of *F. rubra* seedlings[a]

F. rubra seedling age (wk) and bacterial strain or species	Inoculum size[b]			Recovery[c]							
				MA				VC			
	Cell protein (μg)	MA (ng)	VC[d] (10^6 CFU)	Root (ng)[e]	Medium (ng)	Total (ng)	%	Root (10^6 CFU)	Medium (10^6 CFU)	Total (10^6 CFU)	%
12											
Frankia strain Ai17	5.0	55 ± 3	—[f]	10 ± 4 (85 ± 45)	5.6 ± 4.2	16 ± 2	28 ± 4	—	—	—	—
	25	280 ± 20	—	120 ± 9 (980 ± 150)	54 ± 21	170 ± 20	63 ± 9	—	—	—	—
S. griseoviridis	25	330 ± 23	—	0	80 ± 78	80 ± 78	24 ± 23	—	—	—	—
E. agglomerans	330	2,500 ± 250	1,000	0	400 ± 140	400 ± 140	16 ± 6	3.0 ± 2.5	1,100 ± 700	1,100 ± 700	110
Pseudomonas sp.	57	230 ± 10	100	0[g]	67 ± 3[g]	67 ± 3[g]	29 ± 2[g]	11 ± 12	180 ± 130	190 ± 150	190
4											
Frankia strain Ai17	5.0	55 ± 3	—	2.4 ± 2.4[g] (70 ± 70)[g]	19 ± 4[g]	22 ± 2[g]	39 ± 3[g]	—	—	—	—
	25	280 ± 20	—	28 ± 5 (1,600 ± 300)	85 ± 10	110 ± 10	41 ± 5	—	—	—	—

[a] Means ± standard deviations unless otherwise indicated (see footnote *g*); $n = 3$ to 5.
[b] Inoculum sizes were adjusted by cell protein or viable count.
[c] Recovery after 1 day for the 12-week-old seedlings and after 14 days for the 4-week-old seedlings.
[d] VC, viable count.
[e] Values in parentheses indicate MA quantity per root fresh weight (ng/mg).
[f] —, not measurable.
[g] Values are means ± ranges ($n = 2$).

tion method instead of the dissolved organic carbon assay used in the present study, and thus the results may not be directly comparable. The cell protein/organic carbon ratios of 3-week-old Ai1a and Ai17 cells were close to the respective value (0.56 to 0.61 μg/μg) calculated from the results presented by Blom (1) for *Frankia* strain AvcI1 cultured under similar conditions (20 days, with propionate as a carbon source).

Considerable differences between the MA/protein ratios of different bacterial groups were observed. These differences are probably due to different cell wall types with various MA contents or, alternatively, differences in their physiological state. *Frankia* and *Streptomyces* strains, both having a gram-positive cell wall structure, showed significantly higher ratios than the gram-negative strains, as expected. There was also a significant difference between the two *Frankia* strains, Ai1a and Ai17, but further studies with more strains are needed for final evaluation of the variation. The gram-positive *Bacillus* strain unexpectedly showed an MA/protein ratio closer to those of the gram-negative strains than to those of the gram-positive actinomycetes. Whether the result was typical for this strain or was caused by the relatively poor culture medium, for example, is not known.

Bacterial spores differ in many respects from vegetative cells and may also have different MA/protein ratios. This is a potential problem in MA-based quantification if the sporulation stage of bacteria in situ is unknown or even changes during the experiment. Of the bacteria tested, the *Frankia*, *Streptomyces*, and *Bacillus* strains are all capable of sporulation. The strongly sporulating *Streptomyces* culture had an MA/protein ratio well over 10-fold higher than that of a poorly sporulating one, thus making the rhizosphere quantification prone to errors. The *Frankia* Ai17 strain typically produces abundant sporangia both in pure cultures and rhizosphere experiments, whereas the Ai1a strain shows only poor sporulation (16). This difference may partially explain the observed variations in the MA content of older Ai17 cultures. However, the MA/protein ratios seemed to decrease in older cultures rather than increase, in clear contrast to those for *Streptomyces* cultures. This might be due to differences in the spore constituents, either in the MA or protein content.

In our previous rhizosphere experiments (16), we have usually inoculated the axenic seedlings at 4 weeks of age, after which they have been grown for a further 8 weeks and harvested at 12 weeks of age (16). Therefore, 4- and 12-week-old *Festuca* seedlings were used in the present inoculation-recovery experiments to match the plant size and chemical background to the inoculation and harvesting points in the previous studies. During the very short incubation used with the older seedlings to allow bacterial attachment to roots, no significant proliferation of the slowly growing *Frankia* and *Streptomyces* strains was expected and thus the inoculum size was still a valid comparison point. Similarly, the size and photosynthetic capacity of the 4-week-old seedlings were still small, and thus there was no significant *Frankia* growth expected during the 2-week incubation. The recovery of *Frankia* Ai17 from the *Festuca* rhizosphere was, in all cases, clearly below 100%, including also the plantless controls. This suggests that the recovery loss took place during the collection stages, probably because of attachment of bacteria to glassware, including the glass beads, which present a considerable surface area. More vigorous treatment is obviously needed to detach and collect the bacteria completely.

According to the MA assay results, only *Frankia* strain Ai17 out of the four strains studied colonized the *Festuca* roots to a considerable extent. The size of the colonized population seemed to be dependent on both the root biomass and the initial inoculum size. Interestingly, the other actinomycete, *S. griseoviridis*, did not show any detectable colonization although identical inoculum sizes and homogenization procedures were used for both bacteria. This *Streptomyces* strain is used in the commercial biofungicide preparation Mycostop (Kemira Oy, Helsinki, Finland) (10), and the MA assay may be useful in the related rhizosphere studies (6). The time allowed for bacterial colonization in this study was quite short, and the inoculation age (12 weeks) of the seedlings was also different from that in our previous studies, and thus the colonization results are not

fully comparable. However, the viable counts of *Enterobacter* and *Pseudomonas* strains recovered from *Festuca* roots were of the same magnitude as those obtained in earlier studies (16). The reason for the poor correlation between the total recovered MA quantities and viable counts for these two strains remains unclear.

In conclusion, the MA assay was found to be a useful quantification method for *Frankia* strains and other bacteria with high MA contents in pure cultures and in rhizosphere experiments. For bacteria with low MA contents and poor root colonization, the sensitivity of the assay may be inadequate to monitor the root-attached population, unless additional enrichment steps are used. The age and physiological condition of the bacteria, especially sporulation, may considerably alter the MA assay results, which must thus be monitored in parallel with other methods for each strain in order to ensure reliable quantification in rhizosphere experiments.

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